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Selective Methyl Labeling of Eukaryotic Membrane Proteins Using Cell-Free Expression

Rasmus Linser,*[,†,‡] Vladimir Gelev,[⊥,§] Franz Hagn,[†,∥] Haribabu Arthanari,[†] Sven G. Hyberts,[†] and Gerhard Wagner*[,†]

[†]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, United States

[‡]Max-Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany

[⊥]Faculty of Chemistry and Pharmacy, Sofia University, Sofia 1164, Bulgaria

[§]FB Reagents, Cambridge, Massachusetts 02139, United States

[∥]Institute for Advanced Study, Technische Universität München, 85748 Garching, Germany

Supporting Information

ABSTRACT: Structural characterization of membrane proteins and other large proteins with NMR relies increasingly on perdeuteration combined with incorporation of specifically protonated amino acid moieties, such as methyl groups of isoleucines, valines, or leucines. The resulting proton dilution reduces dipolar broadening producing sharper resonance lines, ameliorates spectral crowding, and enables measuring of crucial distances between and to methyl groups. While incorporation of specific methyl labeling is now well established for bacterial expression using suitable precursors, corresponding methods are still lacking for cell-free expression, which is often the only choice for producing labeled eukaryotic membrane proteins in mg quantities. Here we show that we can express methyl-labeled human integral membrane proteins cost-effectively by cell-free expression based of crude hydrolyzed ILV-labeled OmpX inclusion bodies. These are obtained in *Escherichia coli* with very high quantity and represent an optimal intermediate to channel ILV precursors into the eukaryotic proteins.

Many biologically important proteins that are difficult to crystallize but small enough for NMR studies cannot be produced in bacterial systems at sufficiently high levels needed for isotope labeling because they are toxic to the expression host or vulnerable to the bacterial environment. This includes not only human integral membrane proteins but also other factors interfering with bacterial biology. This problem can often be overcome with cell-free expression methods.[1−3] Other important advantages are associated with combinatorial labeling,[4−6] since amino-acid scrambling is not a problem, with unnatural amino acids, post-translationally modified residues, fluorescent probes, or metal-binding tags.[7,8] Particularly studies of membrane proteins benefit from cell-free expression systems, which tend to provide high yields required for NMR and can provide folded protein in the presence of detergents, polymers,[9] or nanodiscs.[10]

Unfortunately, cell-free expression cannot achieve isotope labeling with precursors, such as ^{13}C glucose or more specifically labeled metabolites, since it lacks the full bacterial metabolism for precursor incorporation into proteins. Instead, adding labeled amino acids is needed. Aside from Stereo-Array Isotope Labeling (SAIL),[11] associated with high costs, selective methyl-labeling methods[12−14] have thus not been available for cell-free systems so far. In particular for ^{13}C^{1}H methyl-labeled Ile, Leu, and Val (ILV) in a deuterated background, used extensively for structure determination and dynamics studies of large proteins, no amino acids that would be suitable are commercially available to-date.

Specific methyl labeling largely facilitates structure elucidation for several reasons: Methyls report on hydrophobic contacts crucial for the definition of interfaces between secondary structure elements in the core.[16] They also serve as important reporters in membrane proteins, which are particularly rich in hydrophobic amino acids. NMR studies of large protein complexes as well as detergent- or nanodisc-solubilized membrane proteins require high deuteration levels and benefit from the removal of ^{13}C−^{13}C couplings. Therefore, the use of fully protonated ^{15}N/^{13}C amino acids is not suitable. In contrast, isolated protonated methyls in deuterated background[17] and stereoselective methyl labeling[18] allow detection of long NOE distance restraints, offer slow transverse relaxation rates, and exhibit wide signal dispersion.[19] Isolated methyls are also excellent reporters for internal protein motion[14] and measuring intermolecular NOEs.[20] ILV labeling schemes, well established for bacterial expression using appropriate precursors, are thus highly desirable for cell-free methods. Here, we report on an easy, affordable, and practical procedure for incorporating methyl-labeled ILV amino acids into cell-free-expressed membrane proteins. In general, this procedure is widely applicable to any other residue-specific labeling scheme compatible with heterologous bacterial expression. We demonstrate the potential of this method using the membrane anchor of the transcriptional-activator precursor sterol regulatory element binding protein (SREBP), which regulates cellular cholesterol and fatty acid synthesis.[21] So far bacterial

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overexpression of the anchor was impracticable presumably due to toxicity to *Escherichia coli* or rapid degradation.

ILV labeling in a cell-free expression setup can be achieved using a multistep procedure (see Figure 1). First, commercial

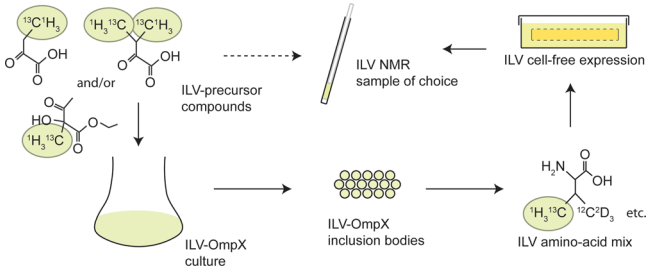

Figure 1. Cell-free-expression procedure for the production of ILV-labeled proteins. A set of commercial precursors is efficiently channeled into ILV-labeled OmpX using *E. coli* expression. Cell-free expression of the ILV-labeled protein of interest is then achieved using the crude amino acids obtained from hydrolyzed inclusion bodies.

ILV precursors are efficiently incorporated into suitable protein biomass. This is achieved by overexpression of the outer membrane protein X (OmpX) in *E. coli*. Here OmpX is overexpressed at very high yield but targeted to inclusion bodies, which makes it harmless to the bacteria. This results in high protein yields of the desired labeling pattern. By growing fully ILV-labeled cell mass containing a high-level expressing protein, the ILV precursors as the limiting resource are efficiently channeled into desired material. In addition to high expression yields, a sufficient amount of ungrouped aliphatic amino acids seems favorable for the inclusion bodies. Hydrolysis of grouped aliphatic amino acids (Val-Val, Ile-Ala, Ala-Ala etc.) requires longer reaction times compared to unproblematic amino acids, presumably due to steric factors and polarity.[22] In OmpX, out of the 28 Ala, Val, Leu, and Ile residues (19% of all residues), only three grouped combinations are formed. After cell harvest, the inclusion bodies are purified by repeated extensive sonication in the presence and absence of Triton-X100. They are then hydrolyzed by treatment with excess methanesulfonic acid at 115 °C for 3 d in the presence of 20% imidazole as a suicide base, similar to experimental conditions used for general amino acid extraction.[23,24] Purification of the hydrolyzed mixture is achieved by cation exchange chromatography using a Dowex AG50W-X8 resin via a pH gradient provided by an acqueous piperidine solution, solvent evaporation and amino-acid redissolution, and filtration of the aqueous solution using a 500 Da membrane. From 1l minimal medium, we obtained around 300 mg of inclusion bodies, which translates into ~200 mg ILV-labeled amino acids. In the last step we employed cell-free protein expression based on the crude ILV amino acids. Out of 9 mL reaction solution (using 300 mg ILV-labeled amino acids) we obtained ~31 mg of precipitated SREBP1 membrane anchor. Cell-free expression of SREBP was pursued according to published procedures[1] using an optimized pIVEX2.4d (Roche Biosciences) construct. Its purification and refolding will be described elsewhere. The obtained yield is similar as achieved with commercially available, uniformly labeled algal mixtures. See Supporting Information (SI) for more details.

ILV cell-free expression was pursued for two exemplary ILV-labeling patterns, i.e., for methyl assignments (providing fully ^{13}C-labeled side chains and backbone) and for NOESY experiments (with ^{13}C only present in the methyls). In the first case, M9 media prepared in ~95% D_2O and containing $^{15}NH_4Cl$ and ^{13}C, ^{2}H-glucose as the sole carbon source was used for OmpX expression. α-ketoisovalerate and α-ketobutyrate as fully ^{1}H/^{13}C-labeled precursors were added before induction. In the second case, we used ^{12}C, ^{2}H-glucose as the carbon source and added α-ketobutyrate and ethyl 2-hydroxy 2-^{13}C-methyl 3-oxobutanoate as methyl-labeling precursors.[18] The latter procedure results in δ-^{1}H/^{13}C-methyl Ile and stereoselective labeling of only the pro-*S*-methyl in Leu and Val in otherwise u-^{2}H/^{12}C labeled OmpX inclusion bodies.[18]

Figure 2A shows the HSQC pattern of the methyl region recorded on a fully ^{2}H, ^{15}N,^{13}C-labeled, ILV-methyl protonated

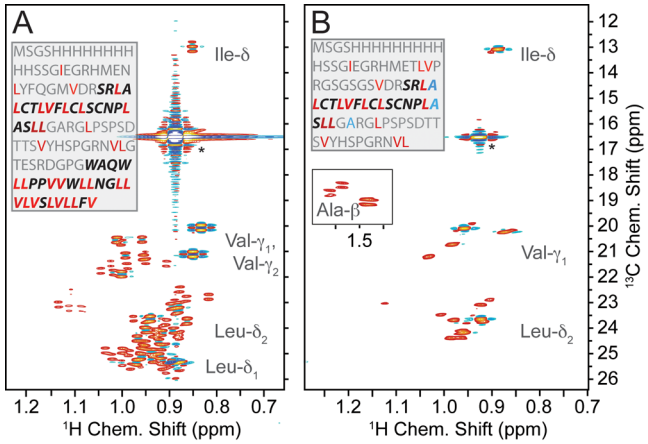

Figure 2. ^{1}H–^{13}C correlated spectra of the methyl region of the ILV-labeled SREBP membrane anchor in LMPG micelles. (A) The full-length anchor with fully ILV-methyl-^{1}H/^{13}C-labeling in otherwise u-^{2}H,^{15}N,^{13}C protein. This construct contains 1 Ile, 10 Val, and 21 Leu residues. (B) The anchor as it remains after S1P cleavage,[15] stereoselectively ILV methyl labeled in an otherwise u-^{2}H,^{15}N labeled background. Additionally, u-^{1}H,^{15}N,^{13}C alanines were incorporated here. The ^{13}C-HSQC spectra were recorded in 3 h on a Bruker 800 MHz spectrometer. Gray annotations denote amino-acid-type assignments. The asterisk marks the natural abundance methyl signal of unlabeled LMPG. Residues with visible methyls are highlighted; bold italics are denoting residues inside the micelle.

SREBP1 membrane anchor. The doublet pattern in the ^{13}C dimension in this case corroborates successful incorporation of this labeling pattern, which is required for commonly used methyl assignment experiments. The strong peak at 0.9 and 16.5 ppm proton and carbon chemical shift, respectively, originates from protonated detergent (lyso-myristoyl glycerol-phosphoglycerol, LMPG) and can be alleviated by deuteration (see Figure 7, SI). Figure 2B depicts a similar spectrum of the anchor as shortened by S1P protease,[15] however largely simplified due to stereoselective methyl Val/Leu labeling, where only one of the two methyl groups is visible. These examples highlight the potential of the presented protocol for membrane protein NMR structure determination. Figure 3 represents an excerpt from a time-shared ^{15}N/^{13}C-edited NOESY,[25] which can be used to obtain structural restraints (measured at 800 MHz in 5 d experimental time).

Cell-free expression is emerging as an important and increasingly popular method for membrane proteins and other challenging targets. The unavailability of methyl labeling for cell-free protein expression, however, has been a crucial limitation for suitable structure-elucidation methods. The

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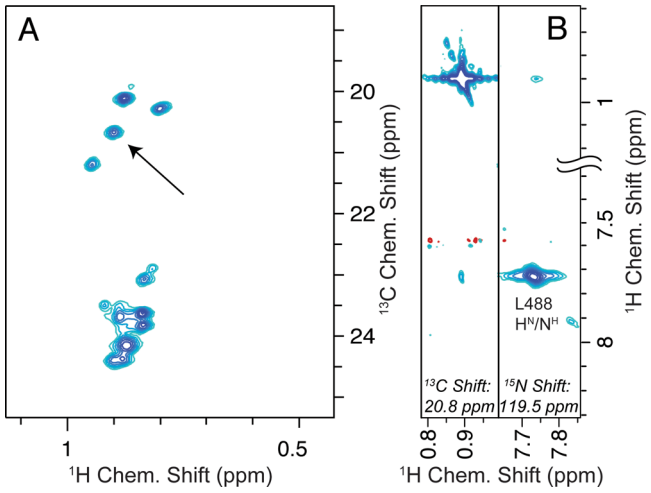

Figure 3. Methyl-amide NOESY contacts obtained for the stereo-ILV-labeled SREBP membrane anchor truncated in the same manner as after native S1P cleavage. (A) Val/Leu region of the first ^{1}H/^{13}C 2D plane as a reference. (B) Strips for an exemplary residue (L488) from the ^{13}C-edited NOESY (left, at an F_2 ^{13}C shift as indicated by the arrow in A) and the matching ^{15}N-edited NOESY strip (right, at L488 ^{15}N^{H} chemical shift). Methyl resonances have not been unambiguously assigned at this point. All of these spectra were obtained simultaneously using a time-shared ^{15}N/^{13}C-edited NOESY experiment[25] at 800 MHz.

described routine is reasonably straightforward and comparably inexpensive and does not require any specialized chemistry equipment besides a rotary evaporator. The feasibility hinges on the high-yield ILV inclusion body expression of the native *E. coli* membrane protein OmpX. This approach may enable structure elucidation of numerous membrane proteins by NMR where commonly used protein production and labeling methods might fail.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details of cell growth, inclusion-body preparation, amino-acid hydrolysis, OmpX data, amino-acid characterization, spectral comparison for different labeling schemes, detergent artifacts. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

rasmus.linser@gmx.de; gerhard_wagner@hms.harvard.edu

Notes

The authors declare the following competing financial interest(s): V.G. declares competing financial interest as founder of fbreagents.com.

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■ REFERENCES

(1) Schwarz, D.; Junge, F.; Durst, F.; Frölich, N.; Schneider, B.; Reckel, S.; Sobhanifar, S.; Dötsch, V.; Bernhard, F. *Nat. Protoc.* **2007**, *2*, 2945.
(2) Spirin, A. S., Swartz, J. R. *Cell-free Protein Synthesis: Methods and Protocols*; Wiley-VCH: Weinheim, 2008.
(3) Klammt, C.; Maslennikov, I.; Bayrhuber, M.; Eichmann, C.; Vajpai, N.; Chiu, E. J.; Blain, K. Y.; Esquivies, L.; Kwon, J. H.; Balana, B.; Pieper, U.; Sali, A.; Slesinger, P. A.; Kwiatkowski, W.; Riek, R.; Choe, S. *Nat. Methods* **2012**, *9*, 834.
(4) Trbovic, N.; Klammt, C.; Koglin, A.; Löhr, F.; Bernhard, F.; Dötsch, V. *J. Am. Chem. Soc.* **2005**, *127*, 13504.
(5) Wu, P. S.; Ozawa, K.; Jergic, S.; Su, X. C.; Dixon, N. E.; Otting, G. *J. Biomol. NMR* **2006**, *34*, 13.
(6) Etzkorn, M.; Raschle, T.; Hagn, F.; Gelev, V.; Rice, A. J.; Walz, T.; Wagner, G. *Structure* **2013**, *5*, 394.
(7) Ugwumba, I. N.; Ozawa, K.; Xu, Z. Q.; Ely, F.; Foo, J. L.; Herlt, A. J.; Coppin, C.; Brown, S.; Taylor, M. C.; Ollis, D. L.; Mander, L. N.; Schenk, G.; Dixon, N. E.; Otting, G.; Oakeshott, J. G.; Jackson, C. J. *J. Am. Chem. Soc.* **2011**, *133*, 326.
(8) Nguyen, T. H. D.; Ozawa, K.; Stanton-Cook, M.; Barrow, R.; Huber, T.; Otting, G. *Angew. Chem., Int. Ed.* **2010**, *50*, 692.
(9) May, S.; Andreasson-Ochsner, M.; Fu, Z.; Low, Y. X.; Tan, D.; de Hoog, H.-P. M.; Ritz, S.; Nallani, M.; Sinner, E.-K. *Angew. Chem., Int. Ed.* **2013**, *52*, 749.
(10) Zocher, M.; Roos, C.; Wegmann, S.; Bosshart, P. D.; Dötsch, V.; Bernhard, F.; Müller, D. J. *ACS Nano* **2012**, *6*, 961.
(11) Kainosho, M.; Torizawa, T.; Iwashita, Y.; Terauchi, T.; Ono, A. M.; Güntert, P. *Nature* **2006**, *440*, 52.
(12) Gardner, K. H.; Rosen, M. K.; Kay, L. E. *Biochemistry* **1997**, *36*, 1389.
(13) Tugarinov, V.; Kay, L. E. *J. Am. Chem. Soc.* **2003**, *125*, 13868.
(14) Sprangers, R.; Kay, L. E. *Nature* **2008**, *445*, 618.
(15) Duncan, E. A.; Davé, U. P.; Sakai, J.; Goldstein, J. L.; Brown, M. S. *J. Biol. Chem.* **1998**, *273*, 17801.
(16) Tugarinov, V.; Choy, W.-Y.; Orekhov, V. Y.; Kay, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 622.
(17) Tugarinov, V.; Kay, L. E. *J. Biomol. NMR* **2004**, *28*, 165.
(18) Gans, P.; Hamelin, O.; Sounier, R.; Ayala, I.; Durá, M. A.; Amero, C. D.; Noirclerc-Savoye, M.; Franzetti, B.; Plevin, M. J.; Boisbouvier, J. *Angew. Chem., Int. Ed.* **2010**, *49*, 1958.
(19) Tugarinov, V.; Sprangers, R.; Kay, L. E. *J. Am. Chem. Soc.* **2004**, *126*, 4921.
(20) Gross, J. D.; Gelev, V. M.; Wagner, G. *J. Biomol. NMR* **2003**, *25*, 235.
(21) Brown, M. S.; Goldstein, J. L. *Cell* **1997**, *89*, 331.
(22) Pickering, M. V.; Newton, P. *LC-GC* **1992**, *8*, 778.
(23) LeMaster, D. M.; Richards, F. M. *Anal. Biochem.* **1982**, *122*, 238.
(24) Hansen, A. P.; Petros, A. M.; Mazar, A. P.; Pederson, T. M.; Rueter, A.; Fesik, S. W. *Biochemistry* **1992**, *31*, 12713.
(25) Frueh, D. P.; Vosburg, D. A.; Walsh, C. T.; Wagner, G. *J. Biomol. NMR* **2006**, *34*, 31.